

Neospora caninum immunoblotting improves serodiagnosis of bovine neosporosis

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Received: 6 March 2006 / Accepted: 13 March 2006 / Published online: 23 May 2006
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Abstract *Neospora caninum* ranges among the major causes of infectious abortion in cattle worldwide. The present study was designed to improve the serodiagnostic tools by complementing a conventional ELISA with a highly sensitive and species-specific *N. caninum* immunoblot. To evaluate this test combination, sera from several groups of cows were tested. The first group, consisting of experimentally infected calves, showed that immunoblot antibody reactivities were detectable 1 to 3 days earlier than those found in ELISA. The first immunodominant bands that appeared were a 29-kDa (NcSAG1) and a 36-kDa (NcSRS2) antigen. Other groups, based upon naturally infected cattle, were used to compare the diagnostic sensitivity of ELISA and immunoblotting. Overall, *N. caninum* immunoblotting exhibited a higher sensitivity (98%) than ELISA (87%). Conversely, immunoblotting also confirm in two other cases, true transient negatigation in some animals. In general, banding patterns and band staining intensity correlated to the semiquantitative ELISA findings. On the other hand, the banding pattern could not be used to discriminate between sera from animals with a recent abortion and those of cows with latent *N. caninum* infection. We also addressed putative cross-reactions due to infection with *Toxoplasma gondii*. Sera from animals with a

serologically proven *T. gondii* infection were either clearly negative by *Neospora* immunoblotting or they yielded a specific immunoblot antibody profile indicating a double infection with *N. caninum*. Sera from animals with positive findings in both *Toxoplasma* and *Neospora* ELISA thus provided dichotomic results in the immunoblot by allowing to confirm or to rule out the specificity of the antibody reaction in *Neospora* ELISA. Altogether, our findings demonstrate that *N. caninum* immunoblotting is a very sensitive and specific complementary tool to improve the serology for *N. caninum* infections in cattle.

Introduction

Neosporosis is one of the most commonly diagnosed causes of infectious abortion in cattle worldwide. In Switzerland, for example, between 25% (Sager et al. 2001) and 29% (Gottstein et al. 1998) of abortions were associated to a *Neospora caninum* infection, as based upon molecular [polymerase chain reaction (PCR)] and pathohistological assessment of the cause of abortion. Two infection routes were described in cattle so far: the endogenous (vertical transmission from the persistently infected mother to the fetus) and the exogenous (horizontal transmission upon uptake of sporulated oocysts or tissue cysts and subsequent passage to the fetus) mode. Several studies have indicated that the endogenous (vertical) transmission occurs in approximately 81–90% of cases (Wouda et al. 2000). This most important route is mainly responsible for the maintenance or spread of an infection in a herd by means of successive passage from generation to generation (Scharles et al. 1998; Piergili Fioretti et al. 2003). Most congenitally acquired infections result in birth of apparently healthy calves, which will become the new carrier; abortion is thus a relatively rare event.

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The influence of the stage of pregnancy on the *N. caninum* distribution and the outcome of infection were shown. Thus, e.g., the parasite DNA detectability in heart and liver was reduced over time of gestation, while that of CNS increased over time. Also, a higher number of positive PCR tissue samples were observed in the fetuses aborted to the first and second pregnancy trimester when compared to the third one. Thus, in the last trimester, the parasite could only be detected in the brain and, sporadically in the diaphragm, heart and lymph nodes (Collantes-Fernandez et al. 2006).

Exogenous (horizontal) transmission was postulated to occur most likely in herds with abortion storms, reflecting a point-source infection (McAllister et al. 1996). In such epidemics, a lack of correlation in the serostatus between dams and offspring was demonstrated (Thurmond and Hietala 1997). Differences in the humoral immune response profile and antibody kinetics may occur upon different routes and doses of infection, especially when discriminating between endogenous and exogenous modes of infection (Gondim et al. 2004).

Seroepidemiological analysis in affected herds may demonstrate statistical association between seropositivity and abortion (Thurmond and Hietala 1997), in that the incidence of abortion appeared approximately four (Davison et al. 1999; Sager et al. 2001) to eight (Vaclavik et al. 2003) times higher when compared to sero- cows. Other groups claimed a protection by high antibody concentrations against abortion due to *N. caninum* (Barling et al. 2000). A definitive diagnosis of neosporosis in cattle implies the appropriate examination of the aborted fetus, e.g., demonstration of the presence of parasite DNA by PCR and revelation of parasite-induced damage by histopathology or immunohistochemistry. However, in many instances, fetal material is not available for laboratory analysis. Therefore, alternative diagnostic approaches are mostly based on serological procedures.

Infection with *N. caninum* gives rise to an antibody response that can be demonstrated by different tests. The presence of antibodies in an animal principally indicates that it remains or was recently infected with the parasite. In cattle, *N. caninum* antibodies were shown to fluctuate during pregnancy and also in between, and they may, at least with some assays, even drop below detection limits (Björkman and Ugglä 1999). Similar observations were done in Switzerland (Sager et al. 2001; Fischer et al. 2003) and a recent work specifically addressed this point (Haesler et al. 2006). Again, for cattle, few attempts were made so far to critically evaluate the specificity of the antibody reactions in view of potential cross-reactions due to *Toxoplasma gondii* or other apicomplexan antibodies (Lally et al. 1996; Chahan et al. 2003). As *T. gondii* was also recognized as an organism infecting cattle and—however,

very rarely—even causing abortion (Gottstein et al. 1998; Canada et al. 2002), differential serodiagnosis appears as a prerequisite to reliably identify the correct parasite species. At the experimental murine level, the presence of cross-reactive epitopes shared by both *N. caninum* and *T. gondii* was documented by immunoblotting and the use of monoclonal antibodies (Liao et al. 2004). Consequently, the serological tools used to diagnose respective infections need to be appropriately validated. We have chosen immunoblot approaches to address this question and to elucidate the species-specificity (and the comparative sensitivity) of serodiagnostic methods. Immunoblotting selection was based upon experiences reported by others. Thus, Schares et al. (1999) used indirect fluorescent antibody test (IFAT) and immunoblotting to address sensitivity and specificity in cattle experimentally infected with *N. caninum*, *T. gondii*, and multiple *Sarcocystis* species. They found that IFAT- and immunoblot-positive aborting dams from herds with endemic *N. caninum*-associated abortions had significantly lower IFAT titers than IFAT- and immunoblot-positive aborting dams from herds with epidemic *N. caninum*-associated abortions. Söndgen et al. (2001) had used immunoblotting to examine fetal fluids for the presence of antibodies against *N. caninum* and found that the diagnostic sensitivity was significantly higher than in IFAT, specificity was also higher than in IFAT if related to histological and PCR findings. Recently, Aguado-Martinez et al. (2005) developed an avidity Western blot to discriminate between acute and chronic infection in cattle. The authors documented that an immunodominant 17-kDa antigen was predominantly responsible for high avidity values.

The aim of our study was to evaluate the operating characteristics of *N. caninum*—immunoblotting under routine diagnostic conditions, thus as to be subsequently able to offer this tool to practitioners for field investigations of abortion problems in Swiss dairy farms.

Materials and methods

N. caninum somatic antigen ELISA

The ELISA system employed was as previously described by Gottstein et al. (1998). The negative/positive threshold value was defined by the mean plus 3 standard errors of 30 sera from cows repeatedly negative in the *Neospora* IFAT and *Neospora* ELISA and derived from farms exhibiting no *Neospora* problem, including a very low or negative seroprevalence for *N. caninum*. The positive control serum was from an experimentally infected calf (Kritznier et al. 2002). Positive serological ELISA results were expressed in

arbitrary antibody units (AU) obtained by linear regression between the cut-off value and that of a highly positive control serum, as previously described (Gottstein et al. 1998). For the test performance, we used antigen plates obtained from Dr. Bommeli AG (Bern), which corresponded to those plates used for the CHECKIT-Neospora sold by the same company. The whole test procedure and control sera used were as previously described (Gottstein et al. 1998). The second antibody (alkaline phosphatase-conjugated rabbit antbovine IgG, Sigma Immunochemicals) was diluted at 1:3,000. Absorbance values were read at $A_{405\text{ nm}}$ (reference $A_{630\text{ nm}}$) using a Dynex MRX II photometer.

T. gondii P30 ELISA

A commercially available, native, affinity purified P30 antigen (ZISR2B, Avril, France) was used for this ELISA, which was basically carried out as described previously (Sager et al. 2003), but employing the same antbovine IgG conjugate as used for the *Neospora* ELISA described above. All other test parameters were identical to those of the *Neospora* ELISA. The negative/positive threshold value was defined by the mean plus 3 standard errors of 30 sera from cows with a repeatedly low reactivity (below 10% of the positive control serum) in the *Toxoplasma* ELISA. Other investigations to etiologically prove the absence of *T. gondii* were not feasible. On the other hand, there was no epidemiological indication that *T. gondii* could have caused any clinical or infectiological troubles on the farms.

N. caninum antigen

The *N. caninum* strain NC-LIV (GB) was used to generate tachyzoites in vitro that were harvested by trypsinization of the vero cell monolayers. Suspension of tachyzoites with cellular debris was sedimented at $1,000\times g$ for 10 min. The pellet was resuspended in phosphate-buffered saline (PBS, pH 7.2), passed through a 25-gauge needle at 4°C, and subsequently separated on a DP10 column filled with Sephadex G-25 (Pharmacia) as described by Hemphill et al. (1996). The purified tachyzoites were stored as a semidry pellet at -80°C until they were used. Frozen tachyzoites were used to prepare the immunoblot antigen exactly as described by Schares et al. (1998, 2001).

N. caninum immunoblot

The immunoblot procedure was basically carried out as described by Schares et al. (1998, 2001). All sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) chemicals were obtained from Fluka (Fluka

Chemie, Buchs, Switzerland). The frozen pellets, each containing 0.66×10^7 *N. caninum* tachyzoites, were resuspended in 80 μl of sample buffer [tris(hydroxymethyl)aminomethane (3.75 g), SDS (10.0 g), glycerol (35.0 ml), bromphenol blue (25.0 g), and the proteinase inhibitor phenyl methyl sulfonyl fluoride (1 mM)] and immediately boiled for 5 min at 95°C . The samples (10 μg protein/cm slot) were electrophoretically separated in a 12.5% gel with a 3% stacking gel. The SDS-PAGE resolved components were electrophoretically blotted onto a sheet of polyvinylidene difluoride (PVDF) (Immobilon-P, pore size 45 μm , lot no. K4HN4464H). Cut PVDF strips were exposed to sera diluted at 1:100 with PBS containing 0.05% Tween 20 and 2% fish gelatin liquid (Serva no. 22156) (henceforth will be called PBS-TG). The strips were incubated for 1 h, while gently shaking, on a warmed up thermoelement (37°C). They were subsequently washed six times with PBS-TG before incubation with antbovine IgG (H+L) peroxidase conjugate (Dianova, cat. no 101-035-003) used at a dilution of 1:300 in PBS-TG for 1 h, while gently shaking, on a warmed up thermoelement. The strips were washed four times with PBS-TG and two times with PBS only. Antigen antibody-reactive bands were visualized using 4-chloro-1-naphthol (Sigma, C-6788) as a precipitating chromogenic substrate. Prestained standard protein ladder markers were from BioRad (Cat. no. 161-0374Rad). The marker section and the first 5 mm of the *N. caninum* antigen part of the PVDF membrane were stained with India ink (Pelikan) solution as described by Hancock and Tsang (1983). To identify antibody reactions against the relevant immunodominant antigens (IDAs), the protocols described by Schares et al. (1998, 1999) and Söndgen et al. (2001) were exactly followed, which was principally based upon the detection of at least two specific bands per strip.

Experimentally infected calf sera

Group 1 Seven calves were experimentally infected with *N. caninum* tachyzoites in analogy with a previous experiment (Kritzner et al. 2002). For the present study, blood samples from days 1, 13, 14, 15, and 27 post infection (p.i.) were used for analysis by ELISA and immunoblotting.

Naturally infected bovine sera

Group 2 For addressing seroepidemiological herd questions, the following sera (obtained from the study published by Sager et al. 2001) were either selected from case farms (i.e., farms exhibiting at least two abortions within the last 4 months or a 20% abortion incidence per year) or from

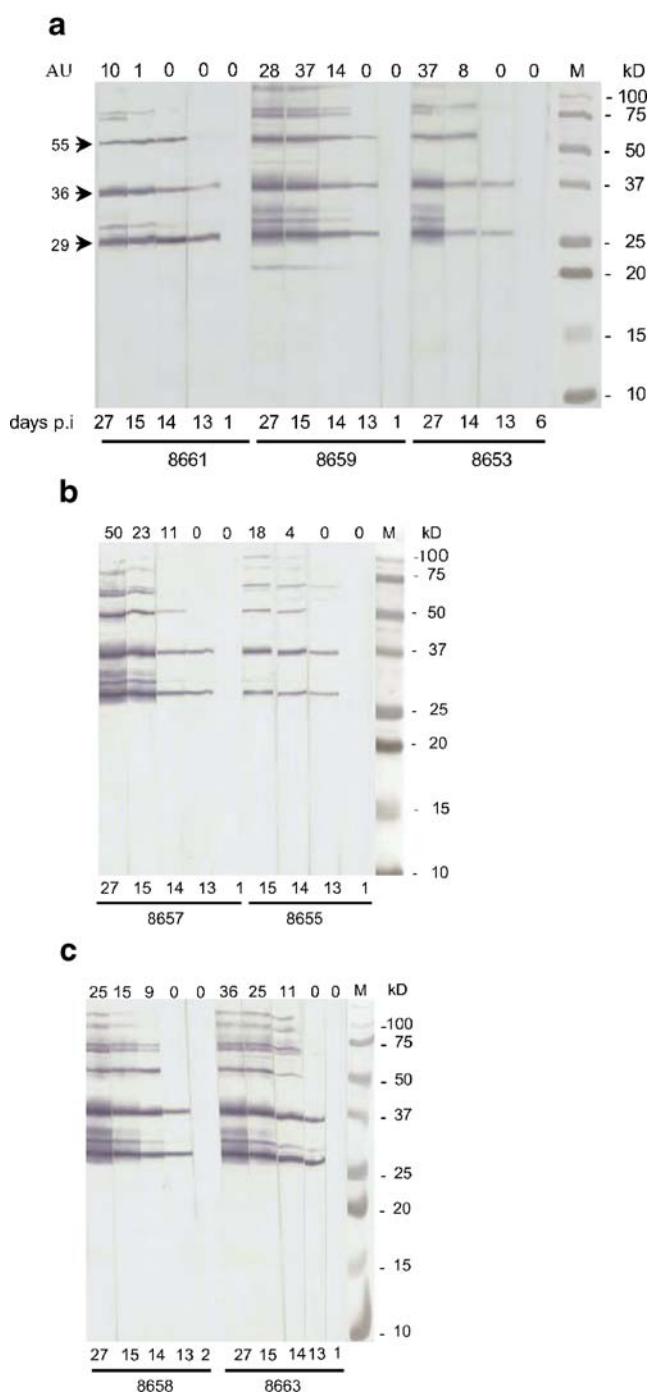


Fig. 1 Immunoblot analyses with sera obtained from seven calves experimentally infected with *N. caninum* (Kritzner et al. 2002) are shown in subpanels **a**, **b**, and **c**, respectively. The four-digit number in the bottom line corresponds to the ID number of each calf. ELISA results are shown on top of lanes and are expressed in arbitrary antibody units (AU). The days post infections are listed at the bottom of each lane. M Molecular marker proteins and their sizes indicated in kilodaltons (kDa). The main immunoreactive bands are indicated by arrows

control farms (i.e., farms of similar size, husbandry, and management system, but without abortion problems):

- Eighteen sera from heifers/cows previously sero+ by *Neospora* ELISA; all animals with abortion history and

the aborted fetal brains being positive by *Neospora* PCR with histopathological evidence of a *Neospora*-induced brain damage;

- Twenty-three sera from heifers/cows previously sero+ by *Neospora* ELISA; all animals with abortion history but the aborted fetal brains being negative by *Neospora* PCR with no histopathological evidence of a *Neospora*-induced brain damage; and
- Ten paired sera from five heifers/cows who showed a seroconversion initially from seropositivity to seronegativity within a 3- to 12-month interval.

Group 3a Sera from 23 cows derived from our routine diagnostic laboratory investigation. These cows had a history of abortion, were sero+ by *N. caninum* ELISA, and their corresponding aborted fetus (brain) tested positive in the *N. caninum* PCR. Furthermore, there was histopathological evidence of a *Neospora*-induced brain damage.

Group 3b Sera from 12 cows derived from our routine diagnostic laboratory. These cows had no history of abortion but were sero+ by *N. caninum* ELISA. This group was complemented with another three sera from cows without history of abortion and a sero- status in the *N. caninum* ELISA (negative controls).

Group 4 Sera from 16 cows all derived from a farm exhibiting an abortion storm history as described by Sager et al. (2005). Eight of these cows exhibited an abortion within a time period of 2 weeks, were sero+ by *N. caninum* ELISA, and their corresponding aborted fetus tested positive in the *N. caninum* PCR. The other eight cows had no history of abortion but were sero+ by *N. caninum* ELISA at the first time point of investigation. For all of these 16 animals, initial avidity ELISA had provided values equal or lower than 25%; sera, thus, were classified as “low avidity,” according to Sager et al. (2005). This indicated a recent exogenous exposure to and infection with *N. caninum* (Sager et al. 2005).

Group 5 Specificity assessment in view of *T. gondii* are as follows:

- Sera from 16 cows derived from our routine diagnostic laboratory with high anti-*Toxoplasma* P30 IgG antibody concentrations, but also simultaneously sero+ in the *Neospora* ELISA and with a *Neospora* PCR-positive aborted fetus (brain).
- Sera from seven cows with high anti-*Toxoplasma* P30 IgG antibody concentrations and simultaneously sero- in the *Neospora* ELISA with a history of abortion and an aborted fetus that tested negative in the *Neospora* PCR without histopathological indication of a *Neospora*-induced brain damage.

Furthermore, we included 17 routine diagnostic sera from aborting cows whose fetuses, however, could not be

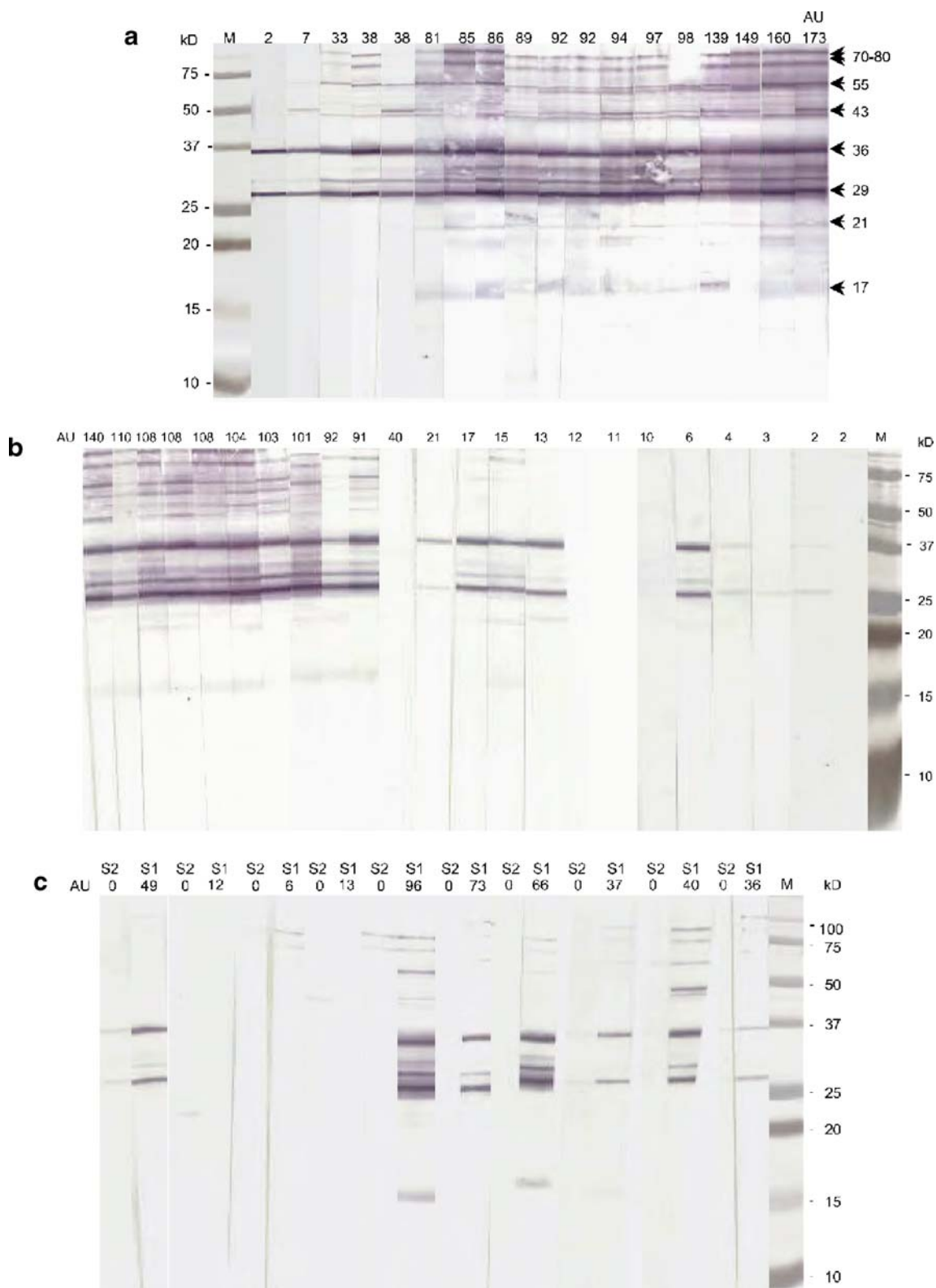


Fig. 2 Immunoblot analyses with sera from different animal groups; corresponding ELISA results are shown on top of lanes and are expressed in arbitrary antibody units (AU). **a** Eighteen *Neospora* ELISA sero+ heifers with abortion history and the aborted fetal brains being positive by *Neospora* PCR with histopathological evidence of a *Neospora*-induced brain damage. **b** Twenty-three *Neospora* ELISA sero+

heifers with abortion history and the aborted fetal brains being negative by *Neospora* PCR with no histopathological evidence of a *Neospora*-induced brain damage. **c** Ten serum samples from five different heifers that showed a seroconversion in the ELISA from initially positive (S1) to negative (S2) within a 3- to 12-month interval. M Molecular marker proteins and their sizes indicated in kilodaltons (kDa)

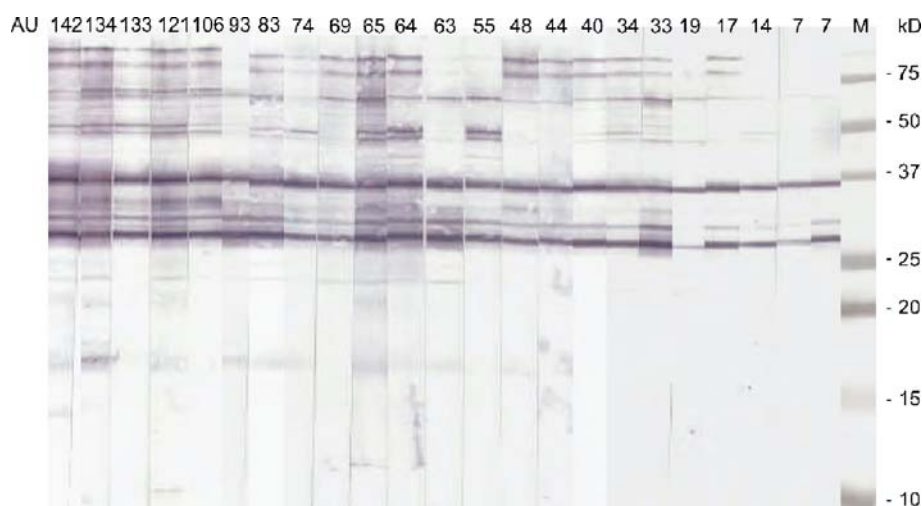


Fig. 3 Immunoblot analyses with sera obtained from our routine diagnostic laboratory, derived from cows with history of abortion, the cows being *Neospora*-positive in the ELISA (mother serum) and the aborted fetus was PCR-positive for *N. caninum*. ELISA results are

shown on *top of lanes* and are expressed in arbitrary antibody units (AU). *M* Molecular marker proteins and their sizes indicated in kilodaltons (kDa)

found by the owner or veterinarian and thus, also could not be investigated by laboratory techniques. These sera were *T. gondii* sero+ and *N. caninum* sero– by ELISA.

Results

Serological findings of group 1 sera

All sera derived from experimentally infected calves showed a temporally increasing antibody concentration in ELISA in correlation with the duration of the infection (data derived from Kritznier et al. 2002). The immunoblot assay provided similar results (Fig. 1a–c) in that a marked increase of immunoreactive band number and intensity was observed during the course of the infection. The first reactive bands appeared as 29 and 36 kDa bands (day 13 p. i.), followed by a 55-kDa band (day 14 p.i.), and subsequently by multiple bands localizing in between these first bands or above in the range between approximately 70 and 80 kDa. Compared with the *Neospora* ELISA, the immunoblot assay appeared more sensitive because the first sero+ reaction was visible on day 13 p.i., whereas *Neospora* ELISA scored positive 1 to 3 days later.

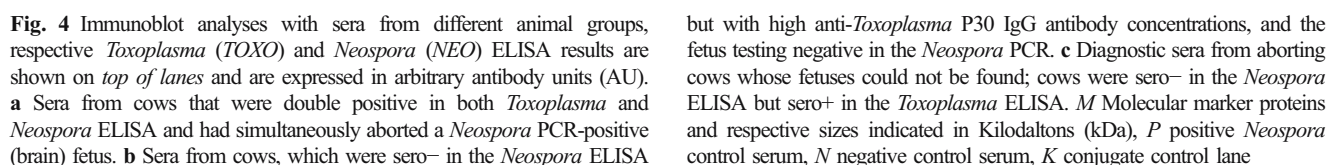
Serological findings of group 2 sera

All of the 18 sera from dams with abortion histories that were sero+ in the *Neospora* ELISA and also *Neospora* PCR-positive for the brain of the aborted fetus exhibited

comparable results in immunoblotting and ELISA (Fig. 2a). ELISA-based immunoreactivities, as expressed in arbitrary AU, correlated with staining intensities and number of bands in the immunoblot assay, which were read and interpreted exactly as indicated by Schares et al. (1998, 1999). The immunoblot banding pattern included the three main bands appearing upon experimental *N. caninum* infection as shown in Fig. 1, plus a series of additional bands such as a 17-kDa band, a 21-kDa band, a 43-kDa double band, and multiple bands between 26 and 33 kDa and around 70–80 kDa.

Investigation of 23 sera from dams that were sero+ in the *Neospora* ELISA but *Neospora* PCR-negative for the fetal brain provided dichotomic results in the immunoblot (Fig. 2b). Four out of 23 samples were negative in the immunoblot, thus indicating false positive reactions in the *Neospora* ELISA. While two of these four sera exhibited only random antibody reactivities in the *Neospora* ELISA reactivity (2 and 3 AU, respectively) and the other two were clearly *Neospora* ELISA-positive (10 and 40 AU, respectively). The immunoreactive banding pattern matched that found in Fig. 2a, including its relation to the degree of ELISA reactivity.

Among ten paired samples from cattle that had presented a *Neospora* ELISA seroconversion from positive to negative between two time points of investigation, six of them were confirmed for seronegativation by immunoblotting (Fig. 2c). In three of the remaining cases, the initial seropositivity (13, 6, and 12 AU, respectively) could not be confirmed by immunoblotting, thus indicating a false positive ELISA finding. In one case, the immunoblot assay



scored positive for both serum samples (first two lanes in Fig. 2c). Also, among these sera, the main reactive bands were the 29- and the 36-kDa band as observed upon experimental infection in Fig. 1.

Serological findings of group 3 sera

Group 3a To increase the number of cases as used in group 2, another 23 sera from cows with history of *Neospora* PCR-positive abortions and seropositivity in the *Neospora* ELISA were included. All sera were also positive in the immunoblot (Fig. 3). Again, ELISA-based immunoreactivities correlated with staining intensities of bands in the immunoblot assay, and the standard banding pattern included all major bands as shown in Figs. 1 and 2.

Group 3b Out of 12 samples from animals without history of abortion but exhibiting seropositivity in the *N. caninum* ELISA, ten scored positive in the immunoblot assay (data not shown). The two negative samples demonstrated only a weak immunoreactivity in the ELISA (10 and 12 AU, respectively).

Searching for qualitative differences in the banding pattern between immunoblotting-positive cows with *Neospora*-induced abortion and those without abortion, no differences could be found.

Serological findings of group 4 sera

All eight aborting cows from a herd experiencing an abortion storm were *Neospora* ELISA-positive at the first time point of investigation, which corresponded to the time period of abortion. Six weeks after abortion, two out of these eight animals became transiently seronegative by ELISA, and another two 3 months after abortion. While all ELISA-positive sera from these eight animals were also positive by immunoblotting, two out of the four ELISA-seronegative sera turned out to be positive by immunoblotting. In general, all of these animals exhibited a basically marked decrease of antibody concentrations after abortion. Another eight nonaborting but *Neospora* sero+ cows (at the time point of abortion of the eight other cows) from the same herd were included as an out-group control. Three from these animals also became transiently sero- by ELISA at one or two time points during a period of 3 months after abortion, but these sera remained all positive by immunoblotting.

Serological findings of group 5 sera

All 18 sera from cows (with a *Neospora* PCR-positive abortion) that were double positive by the *Neospora* and *Toxoplasma* ELISA were also positive in the *N. caninum* immunoblot assay (Fig. 4a), including in most cases, many (but at least the 29 and 36 kDa bands) of the major bands as

described above in the other animal groups. The search for qualitative differences in the banding pattern between double ELISA-positive cows and immunoblot-based serological testing of animals only positive in the *Neospora* ELISA provided no discriminating criteria. Thus, our *Neospora* immunoblot assay alone does not serologically discriminate between these two different infections, but concurrent *Toxoplasma* infection did also not negatively influence the performance of the *Neospora* serology.

The seven cows with *Neospora* PCR-negative abortion histories and *Neospora* ELISA seronegativity, but seropositivity in the *Toxoplasma* ELISA, were all negative in the *Neospora* immunoblot assay (Fig. 4b).

Out of 17 sera from aborting cows (fetus not investigated), which scored positive in the *Toxoplasma* ELISA but negative in the *Neospora* ELISA, 14 were also negative in *Neospora* immunoblotting (Fig. 4c). Three other out of these 17 sera (lanes 4, 13, and 15) showed an weak but specific reaction against the 29 and 36 kDa bands, thus meeting the diagnostic criterion for a *N. caninum*-specific reaction (as described in “Materials and methods”). This indicated a double infection with *N. caninum* not detectable by ELISA.

Operating characteristics

The relative overall diagnostic sensitivity of both test systems (ELISA and immunoblotting) was comparatively assessed by using those sera that showed either one or two positive scorings in ELISA and immunoblotting, respectively. The total number of sera used for these calculations was $n_{\text{tot}} = 131$, the samples were derived from groups 1 ($n = 26$), 2 ($n = 18$), 3a ($n = 23$), 4 ($n = 46$), and 5 ($n = 18$). Immunoblotting yielded 129 positive reactions (relative sensitivity = 98%), ELISA yielded 114 positive reactions (relative sensitivity = 87%).

To calculate the relative specificity, we used the data from 12 sera belonging to groups 3b and 7 plus 17 sera from group 5 ($n_{\text{tot}} = 34$). Using the working hypothesis that immunoblotting provides the gold standard for a specie-specific serodiagnosis, the comparative analysis of immunoblot and ELISA findings results in 29 correct and 5 incorrect ELISA results, thus yielding a relative specificity of 85% for ELISA (compared to 100% for immunoblotting).

Discussion

The objective of this study was to investigate both the sensitivity and specificity of an immunoblot assay suitable to complement conventional serodiagnostic tools of bovine neosporosis. The characterization of operative characteristics should also especially include the serological dis-

crimination between *N. caninum* and *T. gondii* infections in cattle, and the detection of cases with low antibody concentrations that may not be diagnosed with conventional tools.

For the present test assessment, we used different groups of animals or sera to evaluate the operating characteristics: Experimentally infected calves served to comparatively determine the time point of seroconversion after primary infection; groups of naturally infected cows that had been selected upon PCR-positive aborted fetuses were used to comparatively determine the diagnostic test sensitivities upon endogenous transmission; sera from aborting and nonaborting cattle from a farm exhibiting an abortion storm history (exogenous infection mode) were used to determine the diagnostic sensitivity upon horizontal transmission; finally, cattle sera from animals sero+ to *T. gondii* infection served to determine the test specificity in view of potential cross-reactions with the closely related apicomplexan species. The immunoblot interpretation was done according to the criteria defined by Schares et al. (1998, 2001). Reading of the blots included inspection of immunoreactivities of the sera with at least two IDAs. If two or more IDAs were recognized, the sera were considered positive, if less than two were recognized, the immunoblot assay was considered negative. These IDAs are regularly recognized by infected animal species (Barta and Dubey 1992; Bjerkas et al. 1994; Schares et al. 1998).

While compiling the present data available on the diagnostic use of immunoblotting in bovine neosporosis, we realized that there is some discrepancy with regard to the description and documentation of the molecular masses attributed to the major immunodominant antigens appearing on the blots. Schares et al. (1999) and Söndgen et al. (2001) basically listed antigens of 17, 29, 30, 33, 37, and 40 kDa. Shin et al. (2005) reported about six immunodominant bands being recognized by serum IgG of experimentally infected cattle, including 33, 37, 46, 55, 75, and 79 kDa antigens. Our main immunodominant bands localized at 17, 21, 29, 36, 43, and 55 kDa. The two main bands that also appeared first upon experimental infection of calves were the 29 and the 36 kDa bands. To identify the true nature of these antigens, we used affinity-purified rabbit hyper-immune antibodies directed against recombinant NcSAG1 (P30) and against NcSRS2. Anti-NcSAG1 reacted unambiguously with the immunoblot antigen of 29 kDa, anti-NcSRS2 with the 36 kDa antigen. There is a generalized agreement that SDS-PAGE, carried out in different laboratories, will yield slightly different molecular masses due to nonstandardized interlaboratory procedures. Therefore, we assume that our main 29-kDa antigen (NcSAG1), which corresponds well to the 29- or 30-kDa band described by Schares et al. (2001) and Söndgen et al. (2001), might also be to the 33-kDa band described by Shin et al. (2005). The

36-kDa antigen (NcSRS2) appears comparable to the 37-kDa antigen described by Schares et al. (2001), Söndgen et al. (2001), and Shin et al. (2005). Aguado-Martinez et al. (2005) described a different pattern of immunodominant bands; however, their technique was running under reducing conditions, thus rendering the direct comparison of relative molecular mass (M_r) difficult. Nevertheless, their 17-kDa band might correspond well to our 17-kDa band appearing as the lowest M_r band within the whole banding pattern. It may be worthwhile to mention that Howe et al. (1998) identified, characterized, and described the immunodominance of both p29 (NcSAG1) and p35 (NcSRS2) upon the use of mouse and dog sera.

Across all the test runs, immunoblotting showed an overall higher relative sensitivity (98%) when compared to the ELISA (87%). This was particularly demonstrated by testing sera from the experimentally infected calves where immunoblotting became positive earlier than ELISA. The investigation of this group of animals provided very reliable results because other (potentially cross-reacting) infectious organisms could be ruled out, as the animals were raised under high sanitary conditions and underwent continuous health monitoring. ELISA showed an overall lower relative specificity (87%) when compared to the immunoblotting (100%), which was set as gold standard for this parameter. Thus, immunoblotting may be of great help to elucidate unclear ELISA reactions in the context of potential cross-reactions due to, e.g., *T. gondii* or other apicomplexan parasites. Future work will need to investigate sera from bovines experimentally infected with other apicomplexan parasites such as *Sarcocystis* spp. and *Besnoitia besnoiti* to allow more detailed documentation of the specificity of the serological test systems in question. Given our findings in immunoblotting, we could confirm the superiority of its sensitivity and specificity when compared to those of ELISA as previously claimed by Schares et al. (1999) and Söndgen et al. (2001).

Immunoblotting especially proved suitable to elucidate sera that were weakly or borderline reactive in the ELISA (see, e.g., Fig. 2b). This is important because the selection of an appropriate cut-off value in ELISA is critical. When based upon, e.g., a receiver operating characteristic analysis, the final determined threshold represents a compromise between optimal sensitivity and specificity. Thus, low-reactive sera frequently remain “doubtful” with regard to their etiological diagnosis. The high diagnostic value of the immunoblot assay could most convincingly be demonstrated by testing sera from those animals that had been identified by diagnostic PCR as *Neospora*-positive cases. From 18 sera included, 16 exhibited a clear positive result in the ELISA, whereas two showed a borderline result (2 and 7 AU, see Fig. 2a). In this test group, the immunoblot assay demonstrated 100% seropositivity. Overall, among all

sera tested so far for neosporosis in this study, the immunoblot was able to find out 9 sera that were false (although most of them only weakly) positive in the ELISA (but truly negative by immunoblotting) and 11 sera that were false negative by ELISA but truly positive by immunoblotting. Thus, diagnostic sensitivity and specificity were both clearly higher for the immunoblotting procedure.

We also tried to find out if differences in the immunoblot banding pattern could serve as a criterion to serologically discriminate between dams, which had experienced a recent abortion, and cows with latent *N. caninum* infections. Unfortunately, in both cases the banding patterns looked identical, indicating that the immunoblot assay may not be suitable for this particular purpose. This means that conventional immunoblotting cannot discriminate between a new infection and a latent (old) infection. We may, in future, attempt to develop an avidity-based immunoblotting for this specific purpose, as proposed by Aguado-Martinez et al. (2005).

After an abortion storm, antibody concentrations appeared much more subjected to fluctuation than observed in the other animal groups, at least as shown by ELISA. Avidity may hereby play a role, but the infection mode and infection dose may also putatively be responsible for this phenomenon. Fluctuation as shown by ELISA even reaches seronegativity in a few cases. This phenomenon of transient seronegativity in ELISA was already described earlier (Paré et al. 1997; Sager et al. 2001; Haesler et al. 2006). Immunoblotting now revealed that this seronegativity might not be fully true as seven out of nine ELISA negative sera still remained positive by immunoblotting. This could be explained by a lower analytical (methodical) sensitivity of ELISA.

With regard to potential cross-reactivities with sera from *T. gondii*-infected animals, no signals were obtained by immunoblotting. Immunoblotting rather allowed the discrimination of ELISA borderline sera in either clear-cut *Neospora* patterns or into nonspecific patterns (less than two specific bands per lane).

Overall, immunoblotting proved to be a suitable tool to complement conventional ELISA for the serodiagnosis of bovine neosporosis.

Acknowledgements We are grateful to U. Brönniman, P. Stünzi, and C. Wittwer for excellent technical assistance. The study was financially supported by the Swiss Federal Office of Science and Education (BBW C01.0122 in the frame of COST 854), the Swiss Federal Veterinary Office (BVET), and the Department of Clinical Research (Vetsuisse Faculty in Bern). In the frame of international collaboration, a short-term scientific mission of D. S. was financed by COST 854.

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